The chum salmon IGF-II gene promoter is activated by hepatocyte nuclear factor 3β

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Abstract IGF-II plays an important role in growth and development of vertebrates and is highly expressed in adult salmon liver. In the present study, we demonstrate that a liver-enriched transcription factor, hepatocyte nuclear factor 3β (HNF-3β), is an activator of the chum salmon IGF-II gene. Multiple binding sites for HNF-3β were identified within the 5′-UTR using electrophoretic mobility shift assays and mutation of these sites prevents binding of HNF-3β. In transient transfection assays it was shown that mutation of the HNF-3β binding sites results in a substantial decrease of HNF-3β-activated salmon IGF-II gene expression. This is the first identified transcription factor that is functionally involved in the regulation of fish IGF-II expression.

Key words: Insulin-like growth factor II; Gene expression; Hepatocyte nuclear factor 3β; Salmon; *Oncorhynchus keta*

1. Introduction

Insulin-like growth factor II (IGF-II) is a small mitogenic peptide belonging to the insulin family of peptides, sharing common structural features. Members of the insulin family are present in vertebrates, as well as in some invertebrate species. In the two decades that follow the discovery of IGF-II protein [1] massive data on the structure, function and the regulation of expression of mammalian IGF-II genes were obtained (for a review [2]). Only recently was the first fish IGF-II cDNA isolated [3]. Since then, IGF-II cDNAs for other fish species were obtained, and the IGF-II gene structures were characterized for chum salmon [4], barramundi [5], tilapia [6], and rainbow trout [7]. The nucleotide sequence of the pufferfish IGF-II gene has been reported as well (GenBank accession number AL021880). However, the function of IGF-II as well as the regulation of expression of the IGF-II gene in fish are still poorly understood. No transcription factors have been demonstrated to be functionally involved in transcription activation of fish IGF-II genes. High levels of endocrine IGF-II are produced in the liver and thus it seems probable that IGF-II gene expression is activated by liver-specific transcription factors.

The aim of this study is to investigate whether members of the liver-enriched transcription factors family hepatocyte nuclear factor 3 (HNF-3) are capable of enhancing the activity of the promoter of the chum salmon (*Oncorhynchus keta*) IGF-II gene. HNF-3 belongs to the HNF-3/forkhead family of transcription factors, that function as activators of gene expression [8]. Members of this transcription factor family that share a highly conserved DNA binding domain have been described in organisms from yeast to mammals [9]. The zebrafish ortholog of mammalian HNF-3β, *Axial*, can activate the zebrafish sonic hedgehog gene promoter, and interestingly, rat HNF-3β can also activate this promoter suggesting that not only a structural relation between these transcription factors exists, but also a functional homology [10,11]. In the present paper we show for the first time that the liver-enriched transcription factor, HNF-3β, can activate chum salmon IGF-II transcription by binding to multiple sites located in the 5′-UTR of the gene.

2. Materials and methods

2.1. Materials and reagents

Polymerase chain reactions were performed using the PCR kit for Pfu DNA polymerase (Stratagene, La Jolla, CA, USA). Restriction enzymes, T4 DNA ligase, Klenow fragment of DNA polymerase I, and T4 polynucleotide kinase were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden) and New England BioLabs (Beverly, MA, USA). Radioactive isotopes [α-^{32}P]dCTP (3000 Ci/mmol), and [γ-^{32}P]dATP (3000 Ci/mmol) were obtained from New England Nuclear Corp. (Boston, MA, USA).

2.2. Oligonucleotides

Oligonucleotides used for EMSA experiments, PCR, and for mutagenesis were synthesized by Genosys Biotechnologies Inc., UK.

AP3HNF3, 5′-GGACAGCCTCTCACTCAACATCTCTATAGC-3′; AP3RHNF3, 5′-GCTATAGAGATGTTGAGTGAGAGGCTG-TCC-3′; AP4HNF3, 5′-CCTCACCAACTGGGAAACTAACTCAA-CTGC-3′; AP4RHNF3, 5′-GCAGTTGAGTTAGTTTCCCAGTTG-GTGAGG-3′; AP5HNF3, 5′-GCAACCTCTCCAACCAAATATA-ACGCTCAA-3′; AP5RHNF3, 5′-TTGAGCGTTATATTTGGTTG-GAGAGGTTGC-3′; APMAB, 5′-CAGCCTCTCAC**GGGG**CATC-TCTATAGCCCTCACCAACTGGG**GGGG**TAACTCAACT-3′; AP-MABR, 5′-AGTTGAGTTA**CCCC**CCCAGTTGGTGAGGGCTAT-AGAGATG**CCCC**GTGAGAGGCTG-3′; APMBC, 5′-CACCAAC-TGGG**GGGG**TAACTCAACTGCAACCTCTCCAAC**GGGG**TATA-ACGCTCA-3′; APMBCR, 5′-TGAGCGTTATA**CCCC**GTTGGA-GAGGTTGCAGTTGAGTTA**CCCC**CCCAGTTGGTG-3′; APDIR, 5′-GCGCGGATCCGCACTCTCTTTCGCTCTCTC-3′; APREV5, 5′-CGCGCGTTTCCATGTCAATTGGCAGTAATC-3′.

2.3. Plasmid constructs and mutagenesis

The sIGF-II luciferase constructs contain the chum salmon IGF-II gene promoter sequences from −912 to −4 (pSla900) and −299 to −4 (pSla300), relative to the ATG translation initiation codon [4], fused to the firefly luciferase gene-containing expression vector pSla3 [12].

Site-directed mutagenesis of the HNF-3β sites in the sIGF-II-specific sequence was performed according to the manufacturer's instructions, using the Altered Sites II in vitro mutagenesis kit from Promega (Madison, WI, USA). Briefly, a 1410 bp long *Sac*I-*Sac*I fragment of sIGF-II gene sequence from position −912 and extending to the first

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Abbreviations: bp, base pair; IGF-II, insulin-like growth factor II; sIGF-II, chum salmon IGF-II; HNF-3β, hepatocyte nuclear factor 3β; 5′-UTR, 5′-untranslated region

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intron was subcloned into the pALTER vector and used for subsequent mutagenesis. The nucleotide sequences of oligonucleotides used in site-directed mutagenesis are indicated above; nucleotides in bold represent mismatches with the actual sIGF-II sequences. Expression constructs containing the mutated HNF-3β sites were made by subcloning PCR fragments obtained by using primers APDIR and APREV5 into the *Bam*HI and *Nco*I sites of the pSla3 vector. All mutant constructs were checked by sequencing.

For cotransfection experiments we used expression vectors encoding full-length HNF-3α, HNF-3β, or HNF-3γ (kind gifts of Dr. G.R. Crabtree, Stanford University, USA), and for electrophoretic mobility shift assay (EMSA) experiments pGEX-HNF-3β (Dr. R.H. Costa, University of Illinois, Chicago, USA).

2.4. Electrophoretic mobility shift assays

GST fusion protein containing the DNA binding domain of HNF-3β fused to the glutathione S-transferase domain, GST-HNF-3β, was overexpressed in *E. coli* and purified from bacterial extracts using glutathione agarose beads [13]. The DNA fragments used in the EMSA experiments determining the HNF-3 binding sites location were labeled by filling in 5′-protruding ends by using [α-^{32}P]dCTP and Klenow DNA polymerase. Double-stranded (ds) oligonucleotides used in EMSAs were produced by annealing oligonucleotides AP3HNF3 and AP3RHNF3, AP4HNF3 and AP4RHNF3, AP5HNF3 and AP5RHNF3, APMAB and APMABR, APMBC and APMBCR, respectively, and purified from a polyacrylamide gel. Probes were labeled using [γ-^{32}P]dATP and T4 polynucleotide kinase, and purified over a Sephadex G-50 column.

Conditions for EMSA experiments were as described [14]. Briefly, binding reactions were performed by mixing 10^4 cpm of ds DNA probe, competitor DNA and binding buffer, after which protein extract was added. Binding reactions were incubated for 45–60 min on ice. DNA-protein complexes were separated on a 5% (37.5:1 (wt/wt) acrylamide:bisacrylamide) polyacrylamide gel. Gels were dried and exposed on Fuji XR films at −70°C.

2.5. Transient transfections

Transient transfections of Hep3B human hepatoma cells [15] and 293 adenovirus transformed human kidney cells [16] were performed by a modified calcium phosphate procedure using BES buffered saline [17]. Briefly, cells were grown in 25 cm^2 flasks to 60% confluence and transfected with 3 μg of luciferase construct for 293 cells or 4.5 μg of construct for Hep3B cells and 0.5 μg pCMV-LacZ to correct for transfection efficiencies. For cotransfections, pCMV-HNF3β or empty CMV vector was added. Four h after transfection, Hep3B cells were shocked with medium containing 10% dimethylsulfoxide, and both cell lines received fresh medium. Cells were harvested 28 h after transfection and luciferase and β-galactosidase assays were performed as described in [17]. All transfection experiments were performed at least three independent times in duplicate.

3. Results

3.1. HNF-3β transactivates the sIGF-II promoter

Previously, the activity of the salmon IGF-II promoter was assayed in Hep3B and 293 cells, using transient transfection assays. From these results it was determined that maximal promoter activity was present in the pSla900 construct, containing promoter sequences from −912 to −4 relative to the ATG start codon, coupled to the firefly luciferase reporter gene [4]. Using pSla900 in transient cotransfection experiments we examined the putative transactivational properties of three variants of HNF-3: HNF-3α, HNF-3β, and HNF-3γ in the regulation of the sIGF-II promoter. Cotransfection of 293 cells with construct pSla900 and expression vectors encoding either HNF-3α or HNF-3γ resulted in a 1- to 2-fold enhancement of pSla900 activity, whereas HNF-3β stimulated pSla900 activity 6-fold. From these experiments it can be concluded that HNF-3β is an activator of the sIGF-II promoter. To further localize the putative HNF-3β sites additional truncations of the promoter were tested and the smallest construct that still conferred full activation by HNF-3β was pSla300 (Table 1). This suggests that putative HNF-3β responsive elements are located in the first 300 nucleotides upstream of the translation initiation codon of the sIGF-II gene.

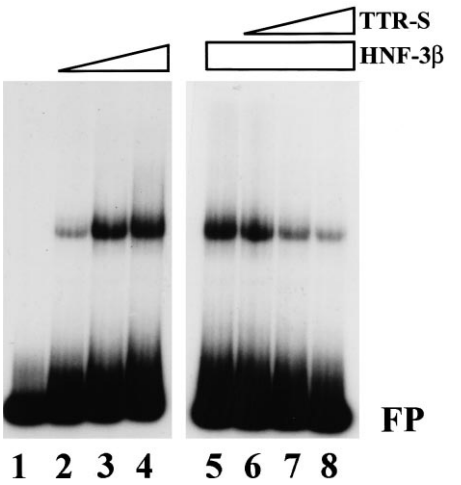

Fig. 1. HNF-3β binds to the proximal promoter region of the sIGF-II gene. Left panel (lanes 1–4): EMSA experiment with GST-HNF-3β protein. Lane 1, probe only; wedges (lanes 2–4) represent increasing amounts of GST-HNF-3β protein. Right panel (lanes 5–8): Competition EMSA with the −299/−4 region of the sIGF-II gene as a probe, a constant amount of GST-HNF-3β protein (lane 5) and increasing amounts of cold competitor oligonucleotide, containing the strong HNF-3 binding site from the TTR promoter (TTR-S) (lanes 6–8). FP, free probe.

3.2. The salmon IGF-II promoter contains three HNF-3 binding sites

To confirm that HNF-3β binds to the region between −299 and −4 relative to the translation initiation codon, electrophoretic mobility shift assays (EMSA) were performed. A 350 bp *Xba*I-*Xba*I DNA fragment from the pSla300 construct representing the region from positions −299 to −4 as well as the first 49 bp of the luciferase gene was used as a probe and incubated with increasing amounts of bacterially expressed GST-HNF-3β. A single complex can be detected (Fig. 1, lanes 2–4), suggesting that HNF-3β forms a complex with this DNA fragment. To confirm that the complex indeed represents binding of HNF-3β, a ds oligonucleotide harboring the high affinity HNF-3 binding site from the transthyretin promoter (TTR-S) [18], was added in increasing amounts in a competition EMSA (Fig. 1, lanes 6–8). TTR-S effectively com-

Table 1
Effects of mutated HNF-3β sites on sIGF-II gene transactivation

Construct	Cell line	
	Hep3B	293
	Fold activation by HNF-3β	
pSla300	2.6 ± 0.3	5.3 ± 0.3
p300mutA	2.4 ± 0.2	3.7 ± 0.3
p300mutBC	1.5 ± 0.2	1.5 ± 0.2

Transient transfection experiments with wild-type (pSla300) and HNF-3β mutant reporter constructs in Hep3B and 293 cells. The basal transcriptional level of each reporter construct was set to 1, and the fold activation by HNF-3β of the wild-type and mutant constructs are indicated. Each number represents the average of at least three independent transfection experiments performed in duplicate. The standard deviation is indicated.

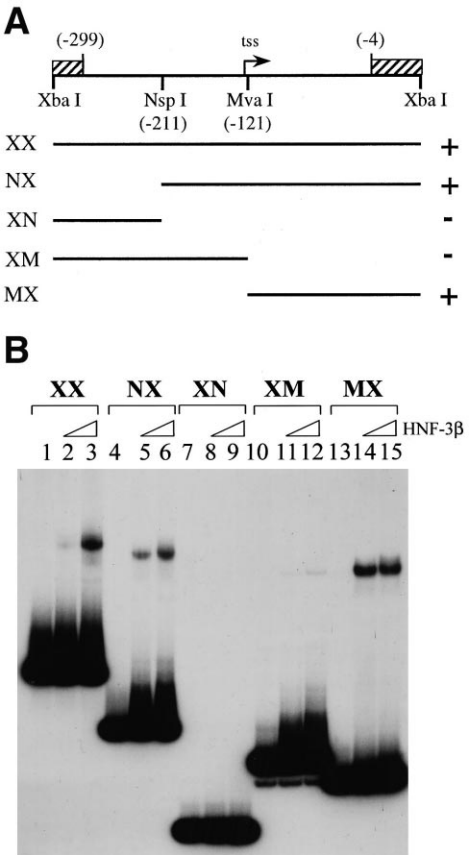

Fig. 2. Localization of HNF-3β binding sites in the sIGF-II promoter by EMSA experiments. A: Top part: Schematic representation of the *Xba*I-*Xba*I fragment from pSla300 containing the region from −299 to −4 of the salmon IGF-II gene relative to the A residue of translation initiation codon. The position of the putative transcription start site (−125) is shown by an arrow and the positions of the *Nsp*I and *Mva*I restriction sites are indicated. The vector-specific parts are shown as striped boxes. Lower part: Fragments used in the EMSA analysis. + indicates that specific DNA-HNF-3β complexes are formed; − indicates that no binding of GST-HNF-3β is observed. B: EMSAs with five sIGF-II-specific fragments. Lanes 1, 4, 7, 10, and 13 contain no protein and wedges indicate increasing amounts of GST-HNF-3β used in EMSAs.

petes with the sIGF-II fragment for HNF-3β binding, indicating that HNF-3β forms a specific DNA-protein complex with the salmon IGF-II promoter region.

To further define the positions of the HNF-3β binding sites, subfragments were tested in similar EMSA experiments (Fig. 2). Using the restriction sites for *Mva*I (−119) and *Nsp*I (−211), respectively, four fragments were gel separated, purified and used in EMSA experiments simultaneously with the whole *Xba*I-*Xba*I fragment (Fig. 2). This experiment shows that the HNF-3β binding sites are located in the most 3′-part of the promoter between positions −119 and +52 relative to the luciferase translation start codon (Fig. 2).

Comparison of the nucleotide sequence of this region with the HNF-3 consensus binding sequence 5′-WRRRYMAA-YA-3′ (W = A/T, R = A/G, Y = C/T, M = A/C), as described by [19], reveals three potential HNF-3 binding sites situated in the sIGF-II-specific part of the *Mva*I-*Xba*I fragment (Table 2). Since all three potential HNF-3 binding sites contained two mismatches to the consensus HNF-3 binding sequence, we checked whether any of these sites was capable of binding to HNF-3β. Three ds oligonucleotides **dsA**, **dsB**, **dsC**, each covering one of the putative binding sites were synthesized and used as probes for HNF-3 binding in EMSA experiments (Fig. 3, lanes 1–9). Incubation of the three probes with increasing amounts of GST-HNF-3β resulted in distinct complex formation. This suggests that all three ds oligonucleotides are capable of binding HNF-3β, and that all three sites may be functional in transactivation of the sIGF-II promoter (Fig. 3).

3.3. Three HNF-3β binding sites are required for maximal activation of the sIGF-II promoter

To establish the functional importance of the three identified HNF-3β binding sites in transactivation of the sIGF-II promoter, the HNF-3 sites were mutated. Four point mutations were introduced in each of the sites as follows: the HNF-3β site A sequence 5′-TCACTCAACA-3′ was altered to 5′-TCAC**GGGG**CA-3′, site B sequence 5′-TGGGAAAC-TA-3′ was altered to 5′-TGGG**GGGG**TA-3′, and site C sequence 5′-CAACCAAATA-3′ was altered to 5′-CAAC**GGG-G**TA-3′. To prove that the introduced mutations could completely abolish HNF-3β binding, EMSA experiments were performed with GST-HNF-3β and the mutated oligos as probes (Fig. 3, lanes 13–18). Mutation of sites A and B (**dsABm**), as well as mutation of sites B and C (**dsBCm**) completely abolished HNF-3β binding. Thus, introduction of the point mutations prevents the formation of HNF-3β-specific DNA-protein complexes (Fig. 3, lanes 13–18).

Finally, to check the functional role of the HNF-3β binding sites, two reporter constructs were made. Because sites B and

Table 2
Interspecies comparison of the transcription start site and the three HNF-3 binding sites of known fish IGF-II genes

Species	Sequence					
		Tss region (−129 to −121)	Site A (−100 to −91)	Site B (−70 to −61)	Site C (−42 to −33)	
Chum salmon [4]	5′-	GCCTGTCGC	TcAcTCAACA	TGGaAAcTA	cAAcCAAATA	-3′
Rainbow trout [7]	5′-	GCCTGTCGC	TcAcTCAACA	TGGaAAcTA	cAAcCAAATA	-3′
Tilapia [6]	5′-	GCCTGTCGC	TcAcTCA*C*CA	TGGaAAcTA	cAAcCAAATA	-3′
Barramundi [5]	5′-	GCCTGTCGC	TcAc*A*CA*T*CA	TGGaAAcTA	cAAcCAAATA	-3′
Pufferfish[a]	5′-	GCCTGTCGC	TcA*TA*CA*T*CA	TGGaAAcTA	c*G*AcCAAAT*C*	-3′
HNF-3 consensus			WRRRYMAAYA	WRRRYMAAYA	WRRRYMAAYA	

Positions of the HNF-3β binding sites of the salmon IGF-II gene are indicated relative to the A residue of the translation initiation codon. Mismatches in the chum salmon sequence to the consensus HNF-3β binding sites are given in lower case. The sequences of the region around the transcription start site and of the three HNF-3β sites from five fish IGF-II genes are compared and differences with the chum salmon gene are indicated (italics). The putative transcription start site is underlined.
[a]Sequence obtained from Genbank, accession number AL021880.

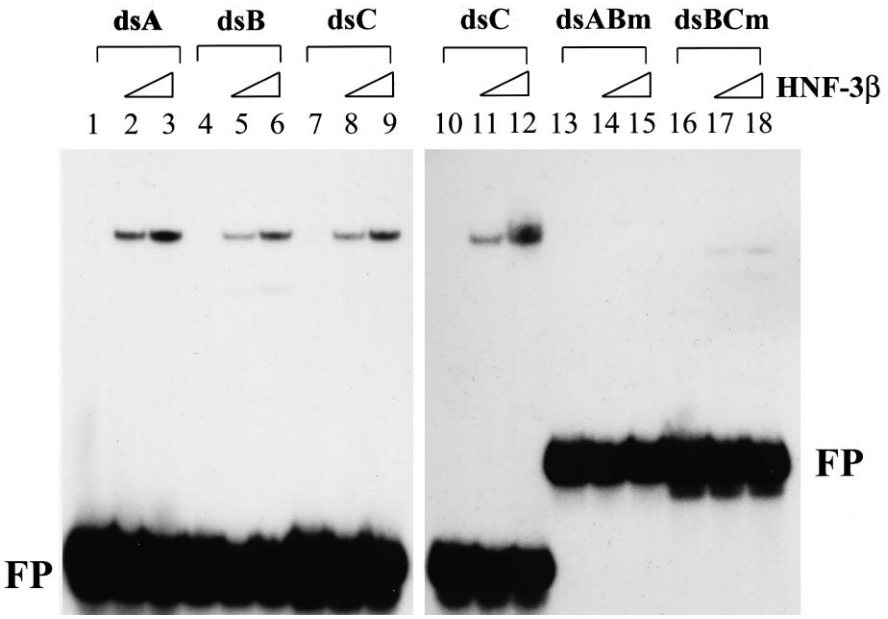

Fig. 3. Mutation of the HNF-3β binding sites abolishes HNF-3β binding. Left panel: EMSAs with ds oligonucleotides each representing one of the three wild-type HNF-3β binding sites (A, B, and C) and increasing amounts of GST-HNF-3β. Lanes 1, 4 and 7: no protein added. Right panel: EMSAs with ds oligonucleotides representing the wild-type HNF-3β binding site C (lanes 10–12), and ds oligonucleotides with mutated sites A and B (lanes 13–15) or mutated sites B and C (lanes 16–18) and increasing amounts of GST-HNF-3β. Lanes 10, 13, and 16, no protein added. FP, free probe.

C are well conserved among different fish IGF-II genes (Table 2), both sites were mutated simultaneously in construct p300mutBC. In addition, to check the contribution of the less conserved site A, a single mutation was introduced in construct p300mutA. The reporter constructs were tested in transient cotransfection experiments with HNF-3β expression vector in 293 and Hep3B cells (Table 1). The transactivation by HNF-3β of the two mutated reporter constructs was lower than that of the wild-type reporter construct in both cell lines tested. While enhancement of the HNF-3β activity was moderately impaired when site A was mutated, a more drastic decrease in HNF-3β transactivation was observed for the mutation of sites B and C. From these results we can conclude that all three HNF-3β sites contribute to the transactivation of the salmon IGF-II promoter by HNF-3β, but that sites B and C have a relatively stronger influence on transcription than site A.

4. Discussion

Previously, we determined the structure of the sIGF-II gene and localized the transcription start site between positions −140 and −110 relative to the A residue of the translation initiation codon [4]. Since then, the characterization of other fish IGF-II genes has been described and the structures and sequences of these genes are highly homologous. Transcription start sites for the rainbow trout IGF-II gene were determined at positions −124 and −125, as well as further upstream [7], and a single transcription start site for the tilapia IGF-II gene was found at the G residue at position −125 [6]. Based on these data and our previous results, and given the extremely high homology of this region (Table 2), it is very probable that the transcription start site of the salmon IGF-II gene is also located at position −125.

In this study, three functional HNF-3β binding sites were determined. It was shown that recombinant HNF-3β binds to the three sites and that mutation of these binding sites completely abolishes the HNF-3β binding. Interestingly, all sites are located within the 5′-UTR of the IGF-II gene. When comparing the nucleotide sequence of the promoter/5′-UTR of the IGF-II gene of several fish species, it is clear that the highest homology is found in the first 200 nucleotides upstream of the translation initiation codon. Only 60 nucleotides upstream of the TATA-box are conserved and further upstream, sequences deviate rapidly. However, of the three functional HNF-3β sites in salmon, two (B and C) are well conserved (Table 2), suggesting that HNF-3β can be a regulator of the IGF-II genes of other fish species as well.

The presence of transcription factor binding sites downstream of the transcription start site is not exceptional. For instance, promoter P3 of the human IGF-II gene was shown to possess a functional binding site for the tumor suppressor gene product WT1 around position +60, relative to the transcription start site. Binding of WT1 represses the transactivation, whereas mutation of the WT1 site enhances promoter activity 2.5-fold [20]. In addition, for the human IGF-I gene two functional HNF-3β sites were identified, one of which is located downstream of the transcription start site [21].

Liver is the major source for IGF-II in salmonids and high IGF-II mRNA levels are maintained into adulthood. In fact, the levels of IGF-II in rainbow trout are two times higher in the adult liver than in the juvenile liver [22]. This suggests the involvement of liver-enriched transcription factors playing a role in the transactivation of the IGF-II gene. In fish, *Axial* is expressed in the very early stage of development, decreases somewhat during later stages of embryogenesis, and was shown to be present in adult zebrafish liver and gut [10]. Additionally, we checked for the presence of *Axial* in adult

salmon liver poly(A) RNA using Northern blotting, and we could confirm the presence of a 1.9 kb *Axial*/HNF-3-specific transcript (data not shown).

To test the ability of HNF-3β to induce activity of the sIGF-II promoter, transfection studies were carried out in the human liver-derived Hep3B cell line and the human 293 kidney cell line. The dedifferentiated Hep3B liver cells do produce some HNF-3β, but introduction of an HNF-3β expression vector is required to fully reconstitute the synthesis of the liver-specific factor HNF-3β. Introduction of the transcription factor and the sIGF-II reporter construct into these cells allows the comparison of the activities of the promoter construct in the presence and absence of HNF-3β. In this study rat HNF-3β was shown to structurally interact and functionally transactivate the salmon IGF-II promoter. The zebrafish ortholog of the HNF-3β transcription factor, *Axial*, was shown to be highly homologous in its DNA binding domain as well as in its transactivation properties [11]. In view of this highly structural and functional conservation among fish and mammals we surmise that the effect of rat HNF-3β on the salmon IGF-II gene promoter will be identical to that of the homologous fish transcription factor *Axial*.

Mutational analysis of the three HNF-3β sites in the sIGF-II reporter constructs was performed and the effects on promoter activity were assayed. Mutation of site A or sites B and C reduced transactivation by HNF-3β to 70% and 25%, respectively in 293 cells, and to 92% and 57% in Hep3B cells. From this we can conclude that mutations in sites B and C severely impair transactivation by HNF-3β, whereas site A mutations only slightly reduce the activation by HNF-3β. The stimulating effect of HNF-3β on sIGF-II transcription is higher in 293 cells than in Hep3B cells. This phenomenon can be explained by the fact that 293 cells do not express any endogenous HNF-3β, whereas Hep3B cells already contain endogenously expressed HNF-3β. Therefore, the fold activation due to cotransfection of exogenous HNF-3β will be lower in Hep3B cells than in 293 cells, since the basal level of promoter activity is already somewhat stimulated by HNF-3β in Hep3B cells.

In summary, in this study we have shown for the first time that a liver-enriched transcription factor can activate sIGF-II gene transcription. This explains, at least partially, the high levels of IGF-II not only during early development, but also in the juvenile and adult salmon.

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